

University of Nebraska - Lincoln DigitalCommons@University of Nebraska - Lincoln

Biochemistry -- Faculty Publications

Biochemistry, Department of

2018

3-O sulfation of heparin leads to hepatotropism and longer circulatory half-life

Colton M. Miller

University of Nebraska- Lincoln, cmiller93@unl.edu

Yongmei Xu

University of North Carolina, Chapel Hill, yongmeix@email.unc.edu

Katrina M. Kudrna

University of Nebraska - Lincoln, kkudrna2@unl.edu

Blake E. Hass

University of Nebraska - Lincoln

Brianna M. Kellar

University of Nebraska- Lincoln

See next page for additional authors

Follow this and additional works at: <http://digitalcommons.unl.edu/biochemfacpub>

 Part of the [Biochemistry Commons](#), [Biotechnology Commons](#), and the [Other Biochemistry, Biophysics, and Structural Biology Commons](#)

Miller, Colton M.; Xu, Yongmei; Kudrna, Katrina M.; Hass, Blake E.; Kellar, Brianna M.; Egger, Andrew W.; Liu, Jian; and Harris, Edward N., "3-O sulfation of heparin leads to hepatotropism and longer circulatory half-life" (2018). *Biochemistry -- Faculty Publications*. 383.

<http://digitalcommons.unl.edu/biochemfacpub/383>

This Article is brought to you for free and open access by the Biochemistry, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Biochemistry -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Colton M. Miller, Yongmei Xu, Katrina M. Kudrna, Blake E. Hass, Brianna M. Kellar, Andrew W. Egger, Jian Liu, and Edward N. Harris

Published in *Thrombosis Research* 167 (2018) 80–87

doi 10.1016/j.thromres.2018.05.018

Copyright © 2018 Elsevier Ltd. Used by permission.

Submitted 16 February 2018; revised 20 April 2018; accepted 15 May 2018;

published 17 May 2018.

3-O sulfation of heparin leads to hepatotropism and longer circulatory half-life

Colton M. Miller,¹ Yongmei Xu,² Katrina M. Kudrna,¹
Blake E. Hass,¹ Brianna M. Kellar,¹ Andrew W. Egger,¹
Jian Liu,² and Edward N. Harris¹

¹ Department of Biochemistry, University of Nebraska, Lincoln, NE 68588, United States

² Department of Pharmacy and Natural Products, University of North Carolina,
Chapel Hill, NC 27099, United States

Corresponding author — E. N. Harris, University of Nebraska,
1901 Vine St, N133, Lincoln, NE 68588, United States; email eharris5@unl.edu

Abstract

Introduction: Heparins are common blood anticoagulants that are critical for many surgical and biomedical procedures used in modern medicine. In contrast to natural heparin derived from porcine gut mucosa, synthetic heparins are homogeneous by mass, polymer length, and chemistry.

Materials & methods: Stable cell lines expressing the human and mouse Stabilin receptors were used to evaluate endocytosis of natural and synthetic heparin. We chemoenzymatically produced synthetic heparin consisting of 12 sugars (dodecamers) containing 14 sulfate groups resulting in a non-3-O sulfated structure (n12mer). Half of the n12mer was modified with a 3-O sulfate on a single GlcNS sugar producing the 3-O sulfated heparin (12mer). Wildtype (WT), Stabilin-1 knock-out (KO), and Stabilin-2 KO C57BL/6 mice were developed and used for metabolic studies and provided as a source for primary liver sinusoidal endothelial cells.

Results & conclusions: Human and mouse Stabilin-2 receptors had very similar endocytosis rates of both the 12mer and n12mer, suggesting that they are functionally similar in primary cells. Subcutaneous injections of the n12mer and 12mer revealed that the 12mer had a much longer half-life in circulation and a higher accumulation in liver. The n12mer never accumulated in circulation and was readily excreted by the kidneys before liver accumulation could occur. Liver sinusoidal

endothelial cells from the Stabilin-2 KO mice had lower uptake rates for both dodecamers, whereas, the Stabilin-1 KO mice had lower endocytosis rates for the 12mer than the n12mer. 3-O sulfation of heparin is correlated to both a longer circulatory half-life and hepatotropism which is largely performed by the Stabilin receptors.

Keywords: Heparin, Liver sinusoidal endothelial cells, Stabilin, Liver, Kidney, Catabolism

1. Introduction

Heparin is a time-honored anticoagulant [1] for use in multiple therapeutic treatments including deep-vein thrombosis [2], kidney dialysis [3], venous thromboembolisms [4], and is used as a key tool to prevent blood coagulation during and after surgery [5]. Heparin is a complex sugar-based polymer composed of N-acetylglucosamine and glucuronic acid/iduronic acid with multiple sulfation sites [6]. The pattern and density of sulfation determines its affinity for the coagulation factors and other proteins and receptors present in the cardiovascular and reticuloendothelial systems [7]. Hence, unfractionated heparin (UFH) has the highest affinity for many of the coagulation factors due to the greater average mass of the polymers (mean mass=13.5 kDa) and its high polydispersity. Low molecular weight heparin (LMWH) consists of the shorter length polymers (mean size=2.5 kDa) as it is prepared from the cleavage products of UFH [8]. LMWH is the most common form of heparin in clinical settings as it may be self-administered post-surgically by the patient and available by prescription [9].

The most important modification of heparin for anticoagulation activity is the 3-O sulfation on *N*-acetylglucosamine. This sulfation is critical for the binding of anti-thrombin III [10] and prevents the processing of pro-fibrin into fibrin. The synthesis of heparin, which has been under development for the past eight years and is now nearly economically feasible, demonstrates that the sulfation of the 3-O position of GlcNS has the most impact on heparin's anticoagulation activity [11–14]. 3-O sulfation is also recognized by the clearance receptors for heparin and may impact anticoagulation timing and activity [15].

Clearance of systemic heparin is through the reticuloendothelial system [16] which involves both liver and kidney function. The clearance of UFH involves liver, in contrast to LMWH which is primarily cleared via the kidneys [17,18]. Patients with renal impairment are subject to increased care and scrutiny with LMWH administration due to kidney accumulation and bleeding complications that may arise [19–21]. The molecular mechanism of heparin clearance is largely mediated by the Stabilin-2 receptor [22] which is expressed in liver, spleen, lymph nodes, bone marrow, and in specialized

structures of the kidney, heart, brain, and eye [23]. Stabilin-2 is a type I receptor and is expressed as two isoforms, a full 315-HARE isoform, and a proteolytically cleaved 190-HARE isoform that retains full functionality as the larger isoform. Stabilin-2 is a constitutively active receptor that uses the clathrin-coat mechanism for rapid internalization with or without ligand [24,25]. Similarly, a close homologue of Stabilin-2, Stabilin-1, is expressed by a wide variety of tissues throughout the vascular and lymphatic systems [26] and may also participate in heparin clearance within the liver [15,27].

In this report, we demonstrate the use of synthetic homogenous heparin (HS) of a defined size (12mer/dodecamer) and compare two polymers which do (12mer) or do not (n12mer) contain a single 3-O sulfation on GlcNS (Fig. 1A) [13,28,29]. These polymers and other similarly sized heparins have been previously used to determine robust binding and internalization by both Stabilin-1 and Stabilin-2 receptors [29]. These receptors are similar to each other [30], though they differ in expression levels, distribution, and affinity for heparin; Stabilin-2 having the highest affinity and higher expression in the liver sinusoids [15,31]. The objectives of this study are to determine the contribution of the 3-O sulfation of the dodecamer (LMWH) in the clearance of homogenous heparins in a WT mouse model and the contribution of these receptors to heparin cellular endocytosis in knock-out mice.

2. Methods and materials

2.1. Animals

Animals were used in accordance with the Institutional Animal Care and Use Committee at the University of Nebraska under breeding protocol #1235 and use protocol #1435. Stabilin-2 knock-out mice were a kind gift from Prof. Atsushi Miyajima at the University of Tokyo and have been previously characterized [32]. The Stabilin-2 knock-out strain was developed in the C57BL/6 background. Stabilin-1 knock-out mice was developed in the C57BL/6 background by Jackson Laboratories using CRISPR/CAS9 technologies to delete the 2nd exon of the gene to produce a non-productive mRNA (supplemental Fig. 1). Both KO strains are homozygous and phenotypically the same as reported previously by Schledzewski et al. [27] and verified by the use of Phire Direct PCR Master Mix (Thermofisher). Male C57BL/6 mice (9–13 weeks old) were lightly anesthetized with 30% isoflurane mixed with polyethylene glycol 200 and injected subcutaneously with 100 μ L of 35 S-labeled heparan sulfate compound mixed in phosphate buffered saline for a final administration of 0.43 mg/kg body weight. Immediately following injection, the mice were placed in a metabolic cage containing food and water ad libitum. At the designated time point, mice were anesthetized with 30% isoflurane and

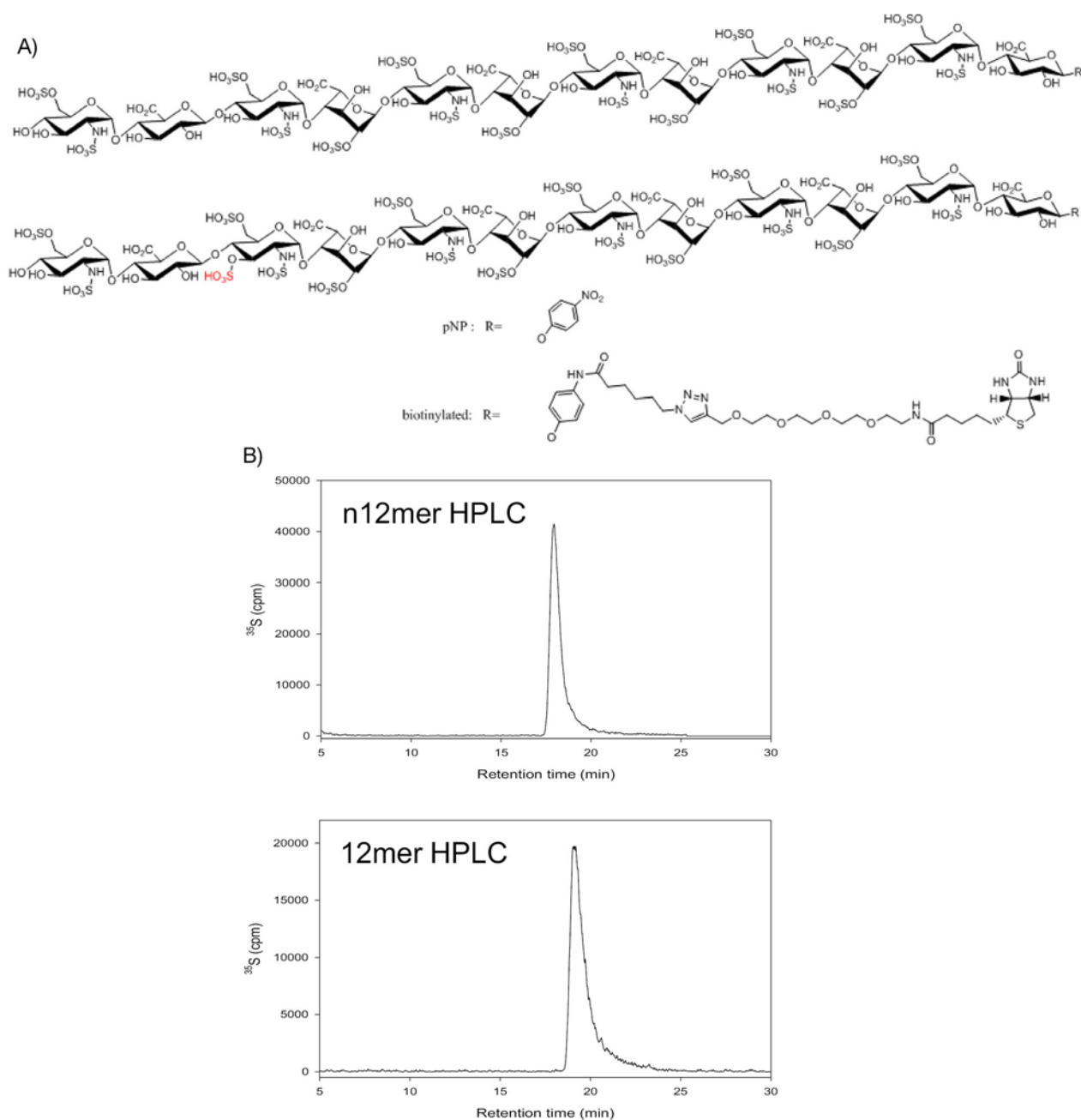


Fig. 1. Structure and purity of the dodecamers. A) Schematic representation of the 3-O sulfated (red highlighted modification) and non-3-O sulfated dodecamers used in this study. B) DEAE-HPLC chromatograms of 12mers. Both 12mers were eluted in a single peak, indicating that the compounds were pure.

blood, urine, liver and kidneys were collected. Blood was centrifuged in a Z serum Sep Clot Activator vacutainer tube (Greiner Bio-one) and 0.1 mL serum was mixed with 4 mL scintillation fluid. Total blood volume was calculated as 7% of body weight by volume. Total urine was collected from both the cage and mouse and 10% of the volume was mixed with 4 mL scintillation fluid and counted. The remaining 90% of urine and remaining serum was retained for the degradation assay. Small pieces of liver, approximately 0.1 g, were cut, weighed, and homogenized in 1% NP-40. Kidneys were cut in half, weighed, and homogenized in 1% NP-40. The homogenized tissues were centrifuged at $6100\times g$ for 2 min to separate out the solids and the liquid fraction was mixed with 4 mL scintillation fluid and counted.

2.2. Synthesis and purification of the ^{35}S -labeled and biotinylated dodecamers

^{35}S -labeled synthetic n12mer was prepared from the synthetic precursor 12mer without 6-O-sulfate groups, $\text{GlcNS-GlcA-(GlcNSIdoA2S)}_4\text{-GlcNS-GlcA-pNP}$ synthesized by following the published method [14,33]. The ^{35}S -sulfate was introduced to the 12-mers by heparan sulfate 6-O-sulfotransferases. Briefly, the reaction consisted of 2-(*N*-morpholino)ethanesulfonic acid (MES) 50 mM, pH 7.0, 6-O-sulfotransferase 1 (6-OST-1) 0.1 mg/mL, 6-O-sulfotransferase 3 (6-OST-3) 0.1 mg/mL and 2 nmol ^{35}S -labeled 3'-phosphoadenosine 5'-phosphosulfate (^{35}S]PAPS) (2×10^7 cpm) and precursor 12mer (2 mg) in a total volume of 0.5 mL. After incubation at 37 °C for 3 h, the 6-O- ^{35}S sulfo group was incorporated into the 12-mer precursor, but the 6-O-sulfation to 12-mer precursor was still incomplete. To this end, an additional round of 6-O-sulfation using unlabeled PAPS to complete the 6-O-sulfation step. Here, MES 50mM pH 7.0 buffer, 6-OST-1 0.5 mg, 6-OST-3 0.5 mg, and 30 μmol unlabeled PAPS were add in a total volume of 0.5 mL, then incubated at 37 °C overnight.

HS 12mers with pNP tag (Fig. 1A) (4 mg) and 0.5 mg Pd/C were dissolved in 20mM NaOAc, pH 5.0 in a total volume of 4 mL. Reaction mixture was vacuumed and refilled with H_2 three times. The reaction was then incubated at room temperature for 4 h. It was then filtered to remove charcoal. The flow through was adjusted to pH 8.5 using 500mM Na_2HPO_4 . Succinimidyl 6-azidohexanoate (8M equivalent of 12mer) was added and incubated at 37 °C overnight. Reaction was purified by DEAE-HPLC column to generate azido tagged 12mers. PBS (pH 7.4) buffer was bubbled using N_2 for 5 min for preparing the sample solution of 0.1M CuSO_4 , 0.1M Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA) (Sigma), 0.15M sodium ascorbate, 0.01M azido tagged 12mer and 0.02M biotin-PEG4-alkyne (Sigma). Mixture of 200 μL THPTA and 40 μL CuSO_4 was vortexed, then 80 μL sodium ascorbate, 100

μ L 12mer and 100 μ L biotin-PEG4-alkyne (from Sigma) was added and bubbled using N_2 for 2 min, then incubated at 37 °C overnight. Reaction was purified by DEAE-HPLC column to generate biotinylated 12mers. HPLC and MS were used to monitor above reactions.

The synthesis of ^{35}S -labeled 12mer was accomplished by converting the non-3-O sulfated 12mer (n12mer) to 12-mer. The reaction consisted of MES 50mM pH 7.0, 10mM $MnCl_2$, 5mM $MgCl_2$, 3-OST-1 0.1 mg/mL and 1 μ mol PAPS and n12mer (1 mg) in a total volume of 1 mL at 37 °C overnight. Both n12mer and 12mer were purified by a diethylaminoethyl (DEAE)-HPLC column [29]. Briefly, the DEAE-NPR column (Tosohaas) was eluted with a linear gradient of NaCl in 20mM sodium acetate buffer (pH 5.0) from 0 to 1M in 60 min at a flow rate of 0.4 mL/ min.

2.3. Isolation and purification of primary liver sinusoidal endothelial cells (LSECs)

The purification of LSECs has been published previously [34,35]. Briefly, an anesthetized mouse had its abdomen exposed and portal vein catheterized with a 24G \times 0.75 in. catheter and the liver was flushed with phosphate-buffer saline at a flow rate of 4 mL/min. The liver was then digested with Type 1 collagenase (Worthington, Lakewood, NJ) at a concentration of 0.5 mg/mL in 45 mL for 12 min or until the volume was completely used up. Differential centrifugation separated hepatocytes from non-parenchymal cells and LSECs were enriched on a 25/50% Percoll gradient, separated from Kupffer cells by selective adhesion on polystyrene plates, washed and plated in collagen-coated 24-well tissue culture polystyrene plates and allowed a 2 h recovery prior to the experiment.

2.4. Cell lines

Established human Stabilin-2 and Stabilin-1 cell lines are described previously [36]. Briefly, the *stab2* cDNA was cloned from human spleen tissue and the *stab1* cDNA was a kind gift from Dr. J. Kzhyshkowska. Both *stab* cDNAs were inserted in pcDNA5/FRT/V5/His6-TOPO (Thermofisher) and named pcDNA5-hStab2 or pcDNA5-hStab1 [15]. The mouse Stabilin-2 cell line used in these experiments was generated in the same manner and has not been previously reported. We have compared it with the human Stabilin-2 cell line in terms of both expression level and receptor activity using a well-established ligand (Supplemental Fig. 2). Stable cell lines were created from 293 Flp-In cells by the transfection of 9.0 μ g pOG44 and 1.0 μ g pcDNA5-hStab2 or pcDNA5-hStab1 or pcDNA5-mStab2 (mouse) and 20 μ L Lipofectamine 2000 in a 100mm dish followed by the addition of selection

media (DMEM+8% FBS+50 µg/mL hygromycin B) 24 h later and stable colonies were selected about 2 weeks post-transfection. Clones were assessed for recombinant protein expression, normal morphology and were β-gal negative (for correct insertion within the FRT site in the genome).

2.5. Degradation assay

The collected degraded samples were run through DEAE column following the procedure described previously [37]. The collected samples were mixed with 1 mL of 0.01% Triton X-100 buffer at pH 5.0 containing 150mM NaCl, 50mM NaOAc, 3M urea, 1mM ethylenediaminetetraacetic acid (EDTA) before they were transferred to a small DEAE column (200 µL in size). The column was then washed three times with the same buffer, 1 mL each wash. The column was further washed three times with 0.25M NaCl in 0.001% Triton X-100 buffer, 1 mL each wash. The column was eluted with 1 mL of 2M NaCl in 0.001% Triton X-100 buffer. The wash flow-through and the eluted samples were collected and the cpm of ³⁵S was measured by a scintillation counter. The eluted samples were analyzed by DEAE-HPLC to determine the elution time of the ³⁵S-labeled peak and compared with the elution time of intact 12mer.

2.6. Endocytosis assay

Endocytosis assays for the ³⁵S-dodecamers and ¹²⁵I-streptavidinbiotinylated dodecamers have been described previously [15,38]. Endocytosis of biotinylated dodecamers was performed with 47 nM ¹²⁵Istreptavidin pre-mixed with 190 nM biotinylated dodecamer. The negative control was incubated with ¹²⁵I-streptavidin alone and subtracted from the experimental values to calculate specific binding/endocytosis. Endocytosis of ³⁵S-dodecamers was assessed to "test" out the reagent on Stabilin-2 expressing cell lines and empty vector control cell lines before it was used on animals. In each case, cells were evaluated in 24-well plates, lysed in 0.3 mL 0.3M NaOH and assessed with a gamma counter for ¹²⁵I or in a scintillation counter for ³⁵S and normalized for protein content using the Bradford assay.

2.7. Statistics

The number of animals used in these experiments was at least 5 per time point and the data was calculated as mean ± standard deviation. Significance was assessed by *t*-test at a 95% confidence level using SigmaPlot 11.2 software.

3. Results

The objective of this study was to determine the contribution of the 3-O sulfate modification of this synthetic LMWH in hepatotropic metabolism. To do this, we used homogenous chemoenzymatically designed heparan sulfate oligomers that were known to interact with the Stabilin receptors [15]. The size of each oligo is 12 sugars long (dodecamer) consisting of 14 sulfate groups arranged as N- and 6-O sulfated GlcNS and 2-O sulfated iduronic acid. One set of oligos was 3-O sulfated on the 1st GlcNS (3rd sugar) from the non-reducing end for a total of 15 sulfated groups and is referred to as the "12mer" in contrast to the "n12mer" which is not 3-O sulfated (Fig. 1A). After the synthesis was complete, each oligo was assessed for purity by HPLC trace (Fig. 1B). The full characterization of their structure by mass spectrometry and NMR in addition with their anticoagulant profile against Factor Xa was previously reported by Xu et al. [29]. Generally, 6-O- [³⁵S]sulfated 12-mers were used for metabolic studies within mice and biotinylated 12-mers were used for cell based assays. The biotin was conjugated to the reducing end of the terminal GlcA via the p-nitrophenol (pNP) linker (in Fig. 1 biotinylated HS) [39].

To verify that the oligos had similar affinities for the human Stabilin receptors, we performed cell-based endocytosis assays with each new preparation of HS oligo. Similar to previously published experiments [29], the oligos that we used have a high affinity for 190-HARE cells which are cells expressing the small isoform of human Stabilin-2 and low affinity for the parent/empty vector cell line (Fig. 2A). We next compared the affinity of the biotinylated 12mer (Fig. 2B) and n12mer (Fig. 2C) labeled with ¹²⁵I-streptavidin with human and mouse Stabilin-2 and human Stabilin-1 in a comparative study to determine that both human and mouse receptors have the same binding/endocytosis activity for the ligand. This data is also useful to determine the validity of our animal models with relevance for heparin metabolism in humans. Both Stabilin-1 and Stabilin-2 lines internalized the oligos with approximately the same rate for 2 h, though accumulation was greater in the Stabilin-2 line over a 6 h time course than in the Stabilin-1 line (data not shown).

To assess the contribution of the 3-O sulfate modification in heparin metabolism, radiolabeled dodecamers with and without the 3-O sulfation were subcutaneously injected in mice at a concentration of approximately 0.43 mg/kg, though the molar ratio of polymers that would act against the coagulation factors is higher in homogeneously prepared samples than in the natural heterogeneous LMWH sample. This dose is similar to a standard injection of 40 mg of LMWH in an 80 kg human patient. Immediately after the injection, individual mice were placed in a metabolic cage for the collection of urine. At each time point, mice were anesthetized and tissues/fluids

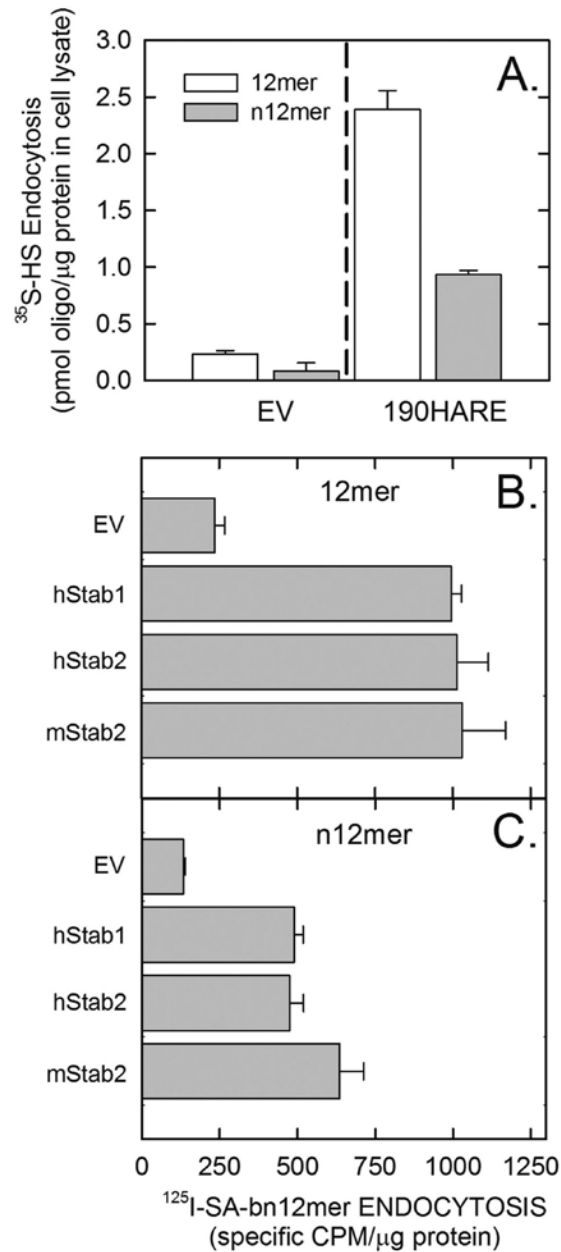


Fig. 2. Internalization of dodecamers in Stabilin expressing cell lines. A) dodecamers labeled directly with ^{35}S were allowed to be internalized by 190- HARE (small isoform of Stabilin-2) expressing cells and empty vector (EV) cells for 3 h. Cells were washed, lysed in 0.3 N NaOH and 90% of the lysate was mixed with scintillation fluid and evaluated by a scintillation counter. The remaining 10% of the lysate was evaluated for protein content by the Bradford assay. B) Biotinylated 12mer and C) biotinylated n12mer were conjugated with ^{125}I -streptavidin and incubated with EV, human Stabilin-1, human Stabilin-2, and mouse Stabilin-2 expressing cell lines for 2 h. Cells were washed 3 times with HBSS, lysed in 0.3 mL 0.3 N NaOH, and evaluated by a gamma counter. Total protein content was evaluated by the Bradford assay. All data presented is the mean \pm standard deviation, $n = 4$.

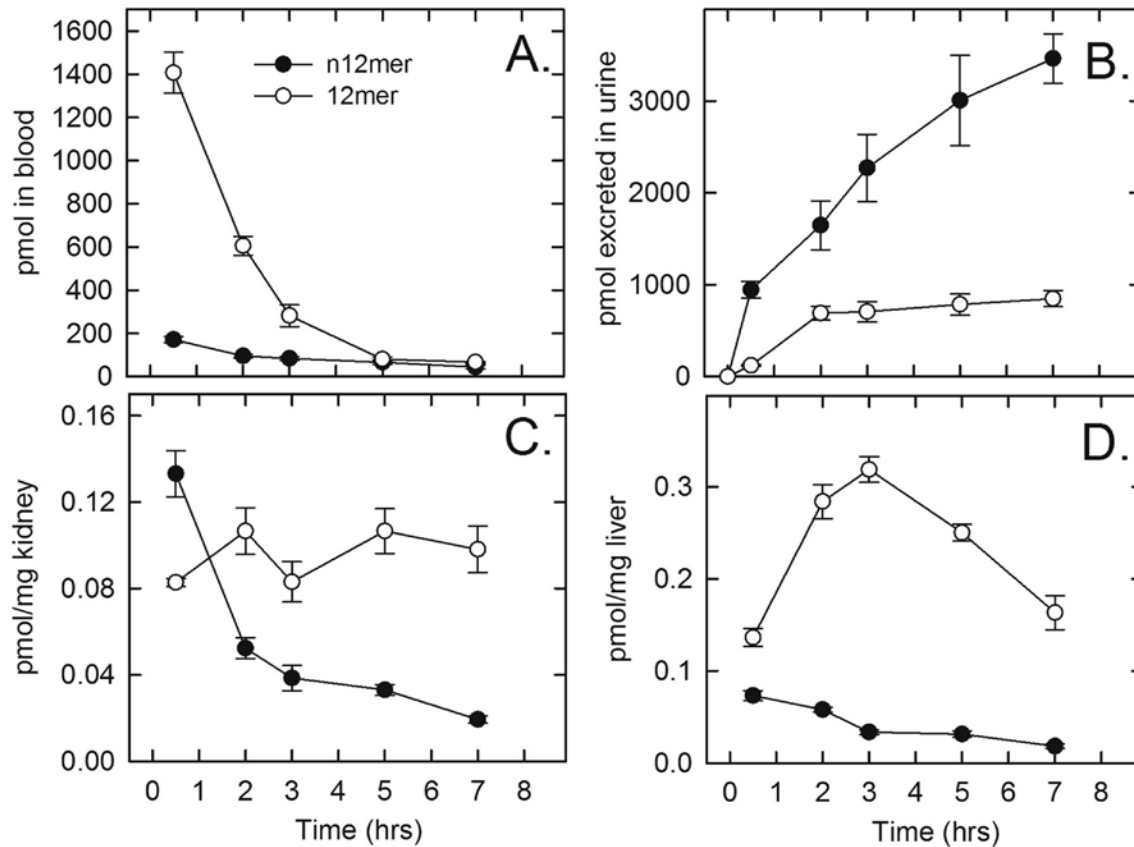


Fig. 3. Rates of accumulation of HS dodecasaccharides in tissues. Mice were subcutaneously injected with ^{35}S -12mer or ^{35}S -n12mer and placed in a metabolic cage until the indicated time point. At the indicated time points, mice were anesthetized and A) serum, B) urine, C) kidney, and D) liver tissues were collected, weighed, and assessed for radioactivity. $n = 5$.

were collected (urine, blood, liver, and kidneys) and assessed for radioactivity and normalized by body weight. The contribution of a single 3-O sulfate made a significant difference in metabolism of these HS dodecamers. In blood, the amount of 12mer spiked initially and then decreased over time with a half-life of about 90 min. The n12mer in blood did not show a significant spike and was cleared from blood roughly as soon as it was perfused in the circulation (Fig. 3A). To determine what had happened to both dodecamers as they were cleared in blood, we obtained urine from the collection cups in the metabolic cages and combined it with any leftover urine in the bladder at the time of sacrifice. The n12mer rapidly accumulated in urine in contrast to the slower rate observed with the 12mer which accumulated slower and then leveled off at the 2 h point (Fig. 3B). The amount of 12mer in kidney stayed in the range of 0.08–0.11 pmol/mg kidney throughout the 7 h time course of the experiment, in contrast to the n12mer which initially

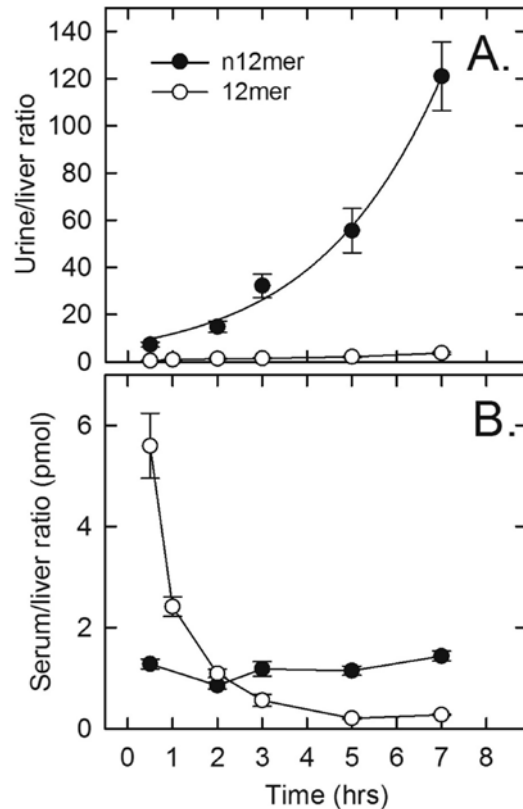


Fig. 4. Accumulation ratios for 3-O sulfated and non-3-O sulfated in tissues. The accumulated radioactivity in either A) urine or B) serum was divided by the liver radioactivity counts at each time point. $n = 5$.

started out at a high of 0.13 pmol/mg and steadily decreased down to 0.02 pmol/mg (Fig. 3C). The accumulation in liver was quite different, in that the 12mer steadily increased and peaked at 0.31 pmol/mg at 3 h and then decreased steadily to 0.16 pmol/mg by 7 h. This is in contrast to the n12mer which peaked at only 0.08 pmol/mg within the first 30 min and slowly decreased to near zero over 7 h.

Next, we examined the data as a ratio of both urine to liver and serum to liver to observe how dynamic the accumulation or clearance of each dodecamer was in relation to the liver. For urine, the amount of n12mer in urine steadily increased over time in contrast to the 12mer which did not increase at all—suggesting that the n12mer is not retained within the liver (Fig. 4A). In serum, the 12mer initially starts very high and decreases at a parabolic rate to near zero at 7 h. This is in contrast to the n12mer which levels off early and maintains a low concentration, suggesting that the n12mer clearance is independent of liver activity (Fig. 4B).

Whether the dodecamers are cleared by kidney or liver, they are degraded over the 7 h time course. To determine the amount of degradation

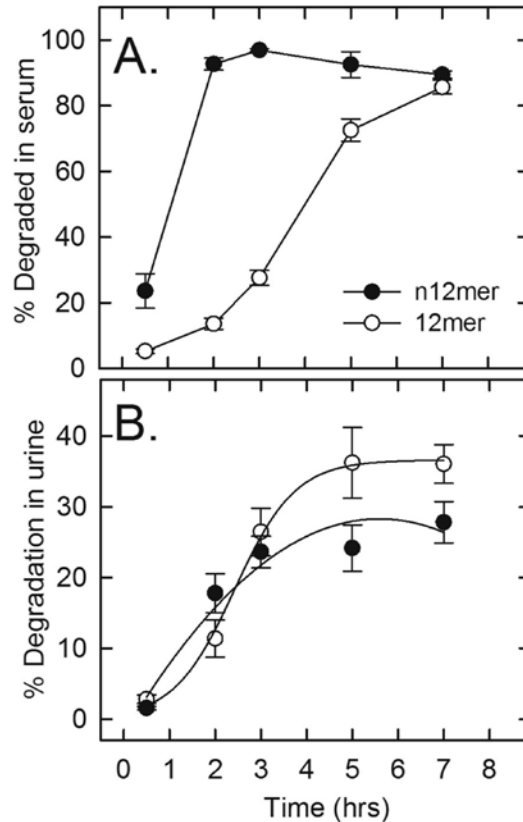


Fig. 5. Degradation of HS dodecasaccharides in urine and serum. At each time point, A) serum and B) urine were collected and fractionated over a DEAE column and counts were assessed by a radiological counter. For serum, each time point is significantly different ($p < 0.01$) except the last (7 h) time point. For the urine, there was no significant difference between the HS oligos at all time points ($p \geq 0.05$), $n = 5$.

in either urine or serum, we ran our samples over a DEAE anion exchange column to separate intact versus degraded polymers. Degraded material is easily washed off in 0.25M NaCl, whereas, intact polymers have a higher affinity for the column and only wash off as the column is regenerated in high salt (2M NaCl). The eluted 12mers from an anion exchange column with 2M NaCl were analyzed by DEAEHPLC. The DEAE-purified ^{35}S -labeled component from urine or serum samples showed the same elution profile on the DEAE-HPLC as that of intact 12mers. The results suggest that the ^{35}S -counts that bound to the anion exchange column represent the undigested 12-mers. Based on the calculation of the ratio of ^{35}S -counts in the wash-off fractions and the eluted fractions, we estimated the amount of degradation of 12-mers. The n12mer had a much higher rate of degradation compared to the 12mer in serum, though both reached the same percentage of degradation by 7 h (Fig. 5A). The percentage of degraded material in urine was

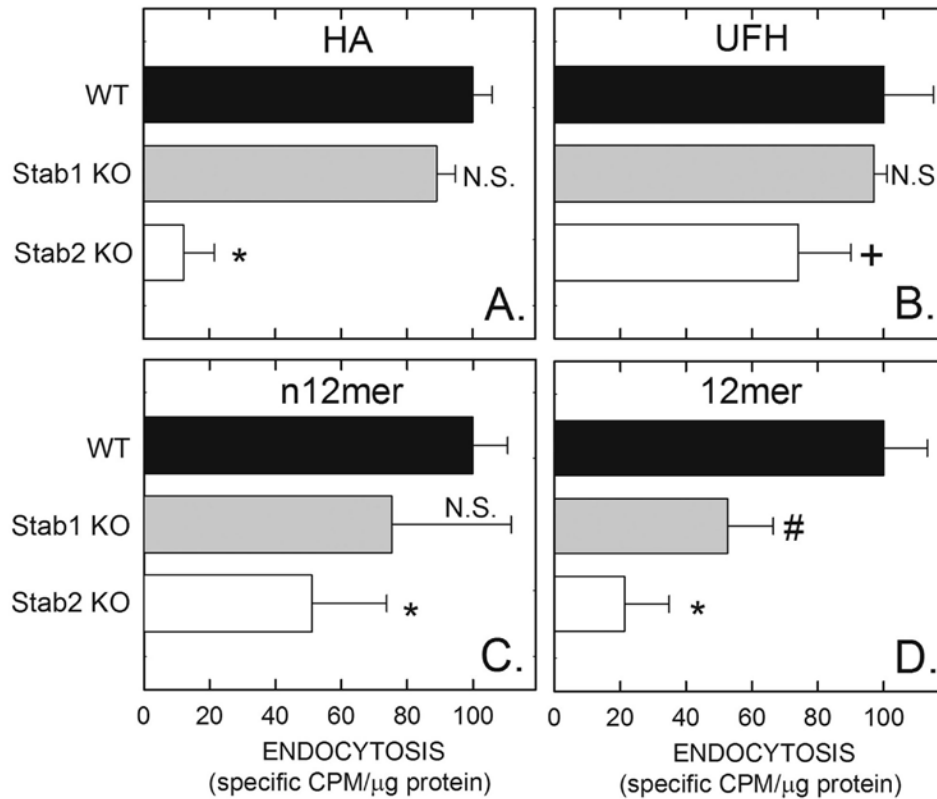


Fig. 6. Endocytosis of ligands by primary liver sinusoidal endothelial cells (LSECs). LSECs from WT, Stab1 KO and Stab2 KO mice were purified, plated on collagen, and allowed to recover for 2 h. Cells were then incubated with either A) ^{125}I -Hyaluronan, B) ^{125}I -SA-b-UFH, C) ^{125}I -SA-12mer and D) ^{125}I -SA-n12mer for 2.5 h, washed 3 times in HBSS, lysed in 0.3 N NaOH and assessed for radioactivity and protein levels by the Bradford assay. Data are the mean \pm standard deviation; $n = 3-6$. N.S. = not significant; * $p < 0.001$; # $p = 0.011$; + $p = 0.08$.

the same for both dodecamers suggesting that clearance mechanisms for clearing dodecasaccharides in kidney is different from liver (Fig. 5B).

Finally, as liver mediated degradation is performed by the liver sinusoidal endothelial cells (LSECs) and not by other cells within the liver [8,40], we purified mouse LSECs from WT, Stabilin-1 KO and Stabilin-2 KO mice and assessed their ability to internalize different ligands. The difference in HA internalization between the WT and Stab1 KO in contrast to the Stab2KO mice is expected ($p < 0.001$) as HA uptake is very specific for the Stabilin-2 receptor (Fig. 6A). In contrast, UFH uptake in WT and Stab1 KO cells was nearly identical and was trending less, though not significantly ($p=0.08$), in the Stab2 KO cells (Fig. 6B). The statistical difference for n12mer uptake between WT and Stab1 KO cells was not significant though there was a statistical difference between WT and Stab2 KO cells (Fig. 6C). The difference in

uptake was significantly higher in both Stab1 and Stab2 KO cells when examined for 12mer endocytosis (Fig. 6D) suggesting that the 3-O sulfate on the HS polymer plays a significant role in the clearance of this molecule via the Stabilin receptors.

4. Discussion

We have demonstrated that the 3-O modification of a heparin polymer changes the metabolic route of the molecule. With this modification, the polymer may bind with coagulation cofactors in the blood and other endothelial cell receptors and molecules to prevent rapid clearance by the kidney. We have also observed that the increased blood residence allows for increased exposure for binding with the Stabilin receptors which allow the heparin to be internalized within the liver. In cell culture, 3-O sulfated 12mers are rapidly taken up by Stabilin expressing cells in contrast to non-3-O sulfated 12mers (this study and [29]). The impact of 3-O sulfation on metabolism is that there is lower accumulation in the kidneys. Although somewhat controversial, one of the common adverse side-effects of LMWH (enoxaparin and not tinzaparin) is bioaccumulation and bleeding that occurs in patients which may be problematic for individuals with renal disease [41,42].

These dodecamers would be classified as LMWH due to their molecular mass and the 3-O sulfated species has a much higher molar ratio of this modification than natural LMWH, potentially allowing for a lower dose to achieve similar bioactivities. Thus, the 12mer is characterized as a heparin species that has the qualities of both LMWH and UFH. The 12mer:Factor Xa (FXa) interaction has about half the activity as the UFH:FXa interaction, but much higher than natural LMWH or Fondaparinux suggesting that the 12mer may be a good candidate to replace natural LMWH as a prophylactic [29]. A more recent study has shown that a similar dodecamer, the super-12mer (s12mer), containing 2 3-O sulfation modifications is similar to Enoxaparin with regards to FXa activity, but is reversible with protamine treatment similar to UFH, thus making it suitable as a heparin analog with enhanced safety [43]. We would argue that a single 3-O sulfation on a 12mer is not quite as potent for FXa activity or neutralization by protamine treatment, but shows us that the contribution of this modification directs the catabolism of the oligo away from immediate filtration by the kidney. We have compared the 12mer and super 12mer (s12mer), a dodecamer with two 3-O sulfated modifications, with regards to binding and endocytosis in the 315-HARE (Stabilin-2) cell lines using the ³⁵S tracer and found the s12mer is typically slightly higher in both categories though the difference may or may not be significant, suggesting that these two polymers are nearly identical in recognition of the Stabilin-2 receptor (Supplemental Fig. 3) [29]. Of course, this is only

relevant with the Stabilin:oligo interaction and may not directly impact FXa activity or other coagulation factors.

The binding site for heparin on the Stabilin receptors is not an obvious string or patch of basic residues and the overall structure for the receptor is unknown at this time. Binding specificity of heparin is not solely due to their high negative charge, but also the specificity of the sugar pattern. For example, we already know from previous work heparin may be competed much better by 4,6-disulfated chondroitin sulfate rather than by 2,6-disulfated chondroitin sulfate even though the overall charge of both CS polymers is the same [38]. Here, we propose that the interaction of the 3-O sulfated GlcNS with the Stabilins has more to do with specificity of this modification within a binding pocket than charge alone. The full-length Stabilin-2 protein has 204 cysteine residues tied up in disulfide linkages and a subset of these are responsible for the folding of 20 epidermal growth factor domains and one X-Link domain [36]. Hyaluronan binds within the X-link domain and is not competed by heparin [38] nor is the X-link domain even required for heparin binding [44], unlike the closest homologous XLink domain in TSG-6 [45]. The dodecamers fulfill the requirement for full binding to the receptor as 10 sugars is the minimum for binding detection [15]. Certainly, the exploration of individual contributions of both Stabilin-1 and Stabilin-2 in the clearance of heparin and other high negatively charged molecules is on-going.

We know that the Stabilin receptors are major clearance receptors for heparin, but are likely assisted by other receptors on the cell surface. Due to their charge, heparins are “sticky” molecules and may bind to a multitude of proteins on the cell surface, but few of those proteins are professional recycling receptors. The Stabilin-2 KO LSECs internalized heterogeneous heparin (UFH) at nearly the same rate as the WT. This may be due to the presence of Stabilin-1 in these cells and other receptors that may respond to the lack of the presence of Stabilin-2. The Stab2KO LSECs had lower internalization rates of both n12mer and 12mer suggesting that Stabilin-2 has a higher affinity for the HS molecule than Stabilin-1. A single knockout of either Stabilin receptor did not significantly impact UFH uptake. There may be two explanations for this. First, the remaining Stabilin receptor may serve to compensate for the loss of the companion Stabilin receptor in these cells. Second, there is a high probability that there are other receptors or molecules on the surface of the LSEC that may bind with heparins [46]. These may or may not be professional recycling receptors, but the cumulative effect is significant for heparin metabolism. When we compare our cell lines which are in the HEK 293 background with LSECs from either mice or rats, the cell lines are much cleaner and demonstrate lower background binding activity (see Fig. 2) than the primary cells. This suggests that there are other molecular factors in the primary cells that work in parallel with the Stabilin receptors.

In our experiments, we demonstrated that endocytosis of both the n12mer and 12mer are significantly impacted by Stabilin-2 suggesting that this receptor is a major contributor for heparin clearance.

5. Conclusions

The 3-O sulfation modification of synthetic homogenous heparin increases half-life in the blood and directs the catabolism to the liver rather than the kidney. Both Stabilin-1 and Stabilin-2 receptors recognize the 12mer with higher affinity than the n12mer and the affinity of these ligands for Stabilin-2 exceeds Stabilin-1.

Acknowledgments — This work was supported by the National Institutes of Health R01HL130864 to E.N.H.

Appendix A. Supplementary data — Supplementary data to this article can be found following the References.

References

- [1] J. Hirsh, T.E. Warkentin, S.G. Shaughnessy, S.S. Anand, J.L. Halperin, R. Raschke, C. Granger, E.M. Ohman, J.E. Dalen, Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety, *Chest* 119 (1 Suppl) (2001) 64S–94S.
- [2] R.D. Hull, G. Townshend, Long-term treatment of deep-vein thrombosis with low-molecular-weight heparin: an update of the evidence, *Thromb. Haemost.* 110 (1) (2013) 14–22.
- [3] J.I. Shen, W.C. Winkelmayer, Use and safety of unfractionated heparin for anticoagulation during maintenance hemodialysis, *Am. J. Kidney Dis.* 60 (3) (2012) 473–486.
- [4] L. Robertson, L.E. Jones, Fixed dose subcutaneous low molecular weight heparins versus adjusted dose unfractionated heparin for the initial treatment of venous thromboembolism, *Cochrane Database Syst. Rev.* 2 (2017) CD001100.
- [5] R. Ikesaka, A. Delluc, G. Le Gal, M. Carrier, Efficacy and safety of weight-adjusted heparin prophylaxis for the prevention of acute venous thromboembolism among obese patients undergoing bariatric surgery: a systematic review and meta-analysis, *Thromb. Res.* 133 (4) (2014) 682–687.
- [6] D.L. Rabenstein, Heparin and heparan sulfate: structure and function, *Nat. Prod. Rep.* 19 (3) (2002) 312–331.

- [7] V.H. Pomin, B. Mulloy, Current structural biology of the heparin interactome, *Curr. Opin. Struct. Biol.* 34 (2015) 17–25.
- [8] E.N. Harris, B.A. Baggenstoss, P.H. Weigel, Rat and human HARE/stabilin-2 are clearance receptors for high- and low-molecular-weight heparins, *Am. J. Physiol. Gastrointest. Liver Physiol.* 296 (6) (2009) G1191–9.
- [9] S. Harder, Renal profiles of anticoagulants, *J. Clin. Pharmacol.* 52 (7) (2012) 964–975.
- [10] M. Petitou, P. Duchaussoy, I. Lederman, J. Choay, P. Sinay, Binding of heparin to antithrombin III: a chemical proof of the critical role played by a 3-sulfated 2- amino-2-deoxy-D-glucose residue, *Carbohydr. Res.* 179 (1988) 163–172.
- [11] U. Lindahl, G. Backstrom, L. Thunberg, I.G. Leder, Evidence for a 3-O-sulfated D-glucosamine residue in the antithrombin-binding sequence of heparin, *Proc. Natl. Acad. Sci.* 77 (1980) 6551–6555.
- [12] U. Lindahl, Approaches to the synthesis of heparin, *Haemostasis* 20 (Suppl. 1) (1990) 146–153.
- [13] U. Bhaskar, G. Li, L. Fu, A. Onishi, M. Suflita, J.S. Dordick, R.J. Linhardt, Combinatorial one-pot chemoenzymatic synthesis of heparin, *Carbohydr. Polym.* 122 (2015) 399–407.
- [14] Y. Xu, S. Masuko, M. Takieddin, H. Xu, R. Liu, J. Jing, S.A. Mousa, R.J. Linhardt, J. Liu, Chemoenzymatic synthesis of homogeneous ultra-low molecular weight heparin, *Science* 334 (2011) 498–501.
- [15] E.H. Pempe, Y. Xu, S. Gopalakrishnan, J. Liu, E.N. Harris, Probing structural selectivity of synthetic heparin binding to Stabilin protein receptors, *J. Biol. Chem.* 287 (25) (2012) 20774–20783.
- [16] E. Adlercreutz, T. Pettersson, Reticuloendothelial system and anticoagulant therapy. Studies with radioactive colloidal gold, *Lancet* 1 (7279) (1963) 470–471.
- [17] P.A. Chindemi, P. Klement, F. Konecny, L.R. Berry, A.K. Chan, Biodistribution of covalent antithrombin-heparin complexes, *Thromb. Haemost.* 95 (4) (2006) 629–636.
- [18] J. Trujillo-Santos, S. Schellong, C. Falga, V. Zorrilla, P. Gallego, M. Barron, M. Monreal, R. Investigators, Low-molecular-weight or unfractionated heparin in venous thromboembolism: the influence of renal function, *Am. J. Med.* 126 (5) (2013) 425–434 (e1).
- [19] W. Lim, Low-molecular-weight heparin in patients with chronic renal insufficiency, *Intern. Emerg. Med.* 3 (4) (2008) 319–323.
- [20] J. Nagge, M. Crowther, J. Hirsh, Is impaired renal function a contraindication to the use of low-molecular-weight heparin? *Arch. Intern. Med.* 162 (22) (2002) 2605–2609.
- [21] M. Crowther, W. Lim, Low molecular weight heparin and bleeding in patients with chronic renal failure, *Curr. Opin. Pulm. Med.* 13 (5) (2007) 409–413.
- [22] E.N. Harris, J.A. Weigel, P.H. Weigel, The human hyaluronan receptor for endocytosis (HARE/Stabilin-2) is a systemic clearance receptor for heparin, *J. Biol. Chem.* 283 (25) (2008) 17341–17350.

- [23] M. Falkowski, K. Schledzewski, B. Hansen, S. Goerdts, Expression of stabilin-2, a novel fasciclin-like hyaluronan receptor protein, in murine sinusoidal endothelia, avascular tissues, and at solid/liquid interfaces, *Histochem. Cell Biol.* 120 (5) (2003) 361–369.
- [24] B. Hansen, P. Longati, K. Elvevold, G.I. Nedredal, K. Schledzewski, R. Olsen, M. Falkowski, J. Kzhyshkowska, F. Carlsson, S. Johansson, B. Smedsrod, S. Goerdts, P. McCourt, Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand binding, *Exp. Cell Res.* 303 (1) (2005) 160–173.
- [25] J. Yannariello-Brown, B. Zhou, P.H. Weigel, Identification of a 175 kDa protein as the ligand-binding subunit of the rat liver sinusoidal endothelial cell hyaluronan receptor, *Glycobiology* 7 (1) (1997) 15–21.
- [26] J. Kzhyshkowska, Multifunctional receptor stabilin-1 in homeostasis and disease, *Scientific World Journal* 10 (2010) 2039–2053.
- [27] K. Schledzewski, C. Geraud, B. Arnold, S. Wang, H.J. Grone, T. Kempf, K.C. Wollert, B.K. Straub, P. Schirmacher, A. Demory, H. Schonhaber, A. Gratchev, L. Dietz, H.J. Thierse, J. Kzhyshkowska, S. Goerdts, Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors, *J. Clin. Invest.* 121 (2) (2011) 703–714.
- [28] R.J. Linhardt, J. Liu, Synthetic heparin, *Curr. Opin. Pharmacol.* 12 (2) (2012) 217–219.
- [29] Y. Xu, C. Cai, K. Chandarajoti, P.H. Hsieh, L. Li, T.Q. Pham, E.M. Sparkenbaugh, J. Sheng, N.S. Key, R. Pawlinski, E.N. Harris, R.J. Linhardt, J. Liu, Homogeneous low-molecular-weight heparins with reversible anticoagulant activity, *Nat. Chem. Biol.* 10 (4) (2014) 248–250.
- [30] O. Politz, A. Gratchev, P.A. McCourt, K. Schledzewski, P. Guillot, S. Johansson, G. Svineng, P. Franke, C. Kannicht, J. Kzhyshkowska, P. Longati, F.W. Velten, S. Goerdts, Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues, *Biochem. J.* 362 (Pt 1) (2002) 155–164.
- [31] R. Prevo, S. Banerji, J. Ni, D.G. Jackson, Rapid plasma membrane-endosomal trafficking of the lymph node sinus and high endothelial venule scavenger receptor/ homing receptor stabilin-1 (FEEL-1/CLEVER-1), *J. Biol. Chem.* 279 (50) (2004) 52580–52592.
- [32] Y. Hirose, E. Saijou, Y. Sugano, F. Takeshita, S. Nishimura, H. Nonaka, Y.R. Chen, K. Sekine, T. Kido, T. Nakamura, S. Kato, T. Kanke, K. Nakamura, R. Nagai, T. Ochiya, A. Miyajima, Inhibition of Stabilin-2 elevates circulating hyaluronic acid levels and prevents tumor metastasis, *Proc. Natl. Acad. Sci. U. S. A.* 109 (11) (2012) 4263–4268.
- [33] R. Liu, Y. Xu, M. Chen, M. Weiwer, X. Zhou, A.S. Bridges, P.L. DeAngelis, Q. Zhang, R.J. Linhardt, J. Liu, Chemoenzymatic design of heparan sulfate oligosaccharides, *J. Biol. Chem.* 285 (2010) 34240–34249.
- [34] S. Gopalakrishnan, E.N. Harris, In vivo liver endocytosis followed by purification of liver cells by liver perfusion, *J. Vis. Exp.* (57) (2011) 3138.

- [35] F. Cabral, C.M. Miller, K.M. Kudrna, B.E. Hass, J.G. Daubendiek, B.M. Kellar, E.N. Harris, Purification of hepatocytes and sinusoidal endothelial cells from mouse liver perfusion, *J. Vis. Exp.* (132) (2018) e56993.
- [36] E.N. Harris, S.V. Kyosseva, J.A. Weigel, P.H. Weigel, Expression, processing, and glycosaminoglycan binding activity of the recombinant human 315-kDa hyaluronic acid receptor for endocytosis (HARE), *J. Biol. Chem.* 282 (5) (2007) 2785–2797.
- [37] Y. Xu, Z. Wang, R. Liu, A.S. Bridges, X. Huang, J. Liu, Directing the biological activities of heparan sulfate oligosaccharides using a chemoenzymatic approach, *Glycobiology* 22 (1) (2012) 96–106.
- [38] E.N. Harris, P.H. Weigel, The ligand-binding profile of HARE: hyaluronan and chondroitin sulfates A, C, and D bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E, *Glycobiology* 18 (8) (2008) 638–648.
- [39] B. Wu, N. Wei, V. Thon, M. Wei, Z. Yu, Y. Xu, X. Chen, J. Liu, P.G. Wang, T. Li, Facile chemoenzymatic synthesis of biotinylated heparosan hexasaccharide, *Org. Biomol. Chem.* 13 (18) (2015) 5098–5101.
- [40] C.I. Oie, R. Olsen, B. Smedsrod, J.B. Hansen, Liver sinusoidal endothelial cells are the principal site for elimination of unfractionated heparin from the circulation, *Am. J. Physiol. Gastrointest. Liver Physiol.* 294 (2) (2008) G520–8.
- [41] M.A. Barras, S.B. Duffull, J.J. Atherton, B. Green, Modelling the occurrence and severity of enoxaparin-induced bleeding and bruising events, *Br. J. Clin. Pharmacol.* 68 (5) (2009) 700–711.
- [42] K.E. Chan, R.I. Thadhani, F.W. Maddux, No difference in bleeding risk between subcutaneous enoxaparin and heparin for thromboprophylaxis in end-stage renal disease, *Kidney Int.* 84 (3) (2013) 555–561.
- [43] M.F. Whelihan, B. Cooley, Y. Xu, R. Pawlinski, J. Liu, N.S. Key, In vitro and in vivo characterization of a reversible synthetic heparin analog, *Thromb. Res.* 138 (2016) 121–129.
- [44] S.V. Kyosseva, E.N. Harris, P.H. Weigel, The hyaluronan receptor for endocytosis mediates hyaluronan-dependent signal transduction via extracellular signal-regulated kinases, *J. Biol. Chem.* 283 (22) (2008) 15047–15055.
- [45] D.J. Mahoney, B. Mulloy, M.J. Forster, C.D. Blundell, E. Fries, C.M. Milner, A.J. Day, Characterization of the interaction between tumor necrosis factor-stimulated gene-6 and heparin: implications for the inhibition of plasmin in extracellular matrix microenvironments, *J. Biol. Chem.* 280 (29) (2005) 27044–27055.
- [46] K.K. Sorensen, J. Simon-Santamaria, R.S. McCuskey, B. Smedsrod, Liver sinusoidal endothelial cells, *Compr. Physiol.* 5 (4) (2015) 1751–1774.

3-O sulfation of heparin leads to hepatotropism and longer circulatory half-life

Colton M. Miller¹, Yongmei Xu², Katrina M. Kudrna¹, Blake E. Hass¹, Brianna M. Kellar¹, Andrew W. Egger¹, Jian Liu², Edward N. Harris¹

¹Dept. of Biochemistry, University of Nebraska, Lincoln NE 68588

²Dept. of Pharmacy and Natural Products, University of North Carolina, Chapel Hill, NC 27099

Supplemental Information

Corresponding Author:

Edward N Harris
University of Nebraska
1901 Vine St, N133
Lincoln NE 68588
402-472-7468
Eharris5@unl.edu

Stab1 Genotyping Assay

Supplemental Fig. 1

#3: Product of length 1011 (rating: 171)
 Contains region of the molecule from 4204 to 5214
 Tm: 81.9 C Tm: 59.2 C GC: 55.4
 Sense Primer:
 GCTGTCCCCATTGCCTGTTATC
 Similarity: 100.0%
 Length: 22 Tm: 57.2 C GC: 54.5
 dH: -170.6 kcal/mol dS: -438.1 cal/mol dG: -38.2 kcal/mol
 Antisense Primer:
 ACTTAAGGATATCGCGGCGC
 Similarity: 100.0%
 Length: 20 Tm: 55.9 C GC: 55.0
 dH: -171.7 kcal/mol dS: -443.0 cal/mol dG: -37.8 kcal/mol
 Tm Difference: 1.3
 GC Difference: 0.5

3647_Stab1_genof: GCTGTCCCCATTGCCTGTTATC
 3648_Stab1_genor: ACTTAAGGATATCGCGGCGC

```

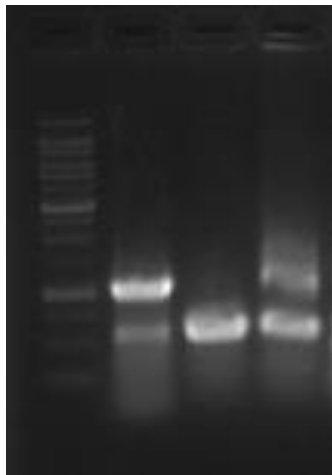
CCCCTGTGCC CCAATTGCCTG TTATCACCAG GGTGCTGGTT CCTATCTGG AGTGCGCGCC TTGGGCCAGC ACTGCCATGG GGCTTTTTC A GCAGTGGCTC
GGGCGACAGG GGTAACGGAC AATAGTGGGC CCACGACCAA GGSATAGACC TCCACCGCGG AACCCGGTGC TGACGGTACC CCGAAAAAGT CGTCACCGAG
CCAGCCCTAA CATGGCAGGC CAGACCTTCT GCTTCGAGCT GGTGCTTGGC GGAAGGCCCTG TGTTTTGTC TAGTGTGACA GGTTGAGTAT GAAATCACTG
GGTCGGGATT GTACCGTCCG GCTCGGAAGA CGAAGCTCGA CCACGAACCG CCTTCGGAC ACAAAACAGG ATCACACITG CCAACTCATA CTTTASTGAC
TACCCCTGTA CACAGTGTGA GGCCTGTGAC GTGTCTGTTT GGACCAAGAA GGCAGGAGC TCATTACCTT GCTTTACACC CTGTCCCTGT GACAGTTTGG
ATGGGGACAT GTGTACACCT CCGAACACTG CACAGACAAA CCTGGTCTT CCGTCGCTCG AGAATAGGGA CGAAATGTGG GACAGGGACA CTGTCAAACC
GACTTCAAGC TCCTCCCTCT AGATGGAAAT GATTCTCTG GAGAAAGAGA AAACCTGAAG GCTTTCAGCC ACAGACAATG GGCAGGGAGG TGACAACGAG
CTGAAGTTCG AGGAGGGGAA TCTACCTTTA CTAAGGAGAC CTCCTTCTCT TTTGGACTTC CGAAAGTTCG TGTCTGTACC CCTGTCTCC ACTGTTGCTC
GTTTCCTAGG ACCTTCCTCT GCCTTGTCTA TTCCCACTGA ACTGAAGGGA AACTGTGCAG TTGTTATAGA CTCAGGCCCC TCACTGTGCA TTTTGCCTAT
CAAAGGATCC TGGAAAGGAC CGGAACGAGT AAGGGTGACT TGACTTCCTT TTGACACGTC AACCATATCT GAGTCCGGGG AGTGACACGT AAAACSGATA
GTCCAGSTA CGGTCTAAAC GTTGCACAT CCACACCAAG TTTGTCACTC ACACACCTGT TACGGCGTGT GCAGCTATCA GAGGCAACT ATGTCCCTGG
CAGGCTCAT GCCAGATTG CAACGCTGTA GGTGTGGTTC AAACAGTGGT TGTGTGGGAC TGTGTGGGAC ATGCCGACA CGTCGATAGT CCTCCGTGA TACAGSGCC
GGTGGTCTC GGAATTTTCC AGAGAAGATA CTTCTGGACT GCGGTATAG CTCCACTCGC TTTTACAC TTTCCGGAGG GTGGCGGGA ACCCAGAGG
CCGACCAAG CTTTAAAGG TCTCTCTAT GAAGACCTGA CCGCATACC GAGGTGAGCG AAGAAATGTG AAAGGCCCTC CACCGCCCTT TGCGTCTCC
CAGCGGGGGA CCCAGAGAAA CCTCACCGAC CTCCAAGTGG GAGCAACAA AGTATCCGGT AACCCACAAA CCTGCTGTG CCTACAGGC AGTAGGCTCA
GTGCGCCCTT GGGTCTCTTT GGAGTGGCTG GAGGTCCACC CTCGTTTGT TCAATGGCCA TTGGGTGTTT GGACGACACA GGATGTCTCG TCATCCGAGT
GGAAAAAGAG AGGAAGGGT TTTAACAAG CACCTACTGT GTGCTGGGCC CTGGGCTCAG CTCGTGGTGA GTTGGAGAGG AGGAGAGAGA TGGGTAGATT
CCTTTTCTCC TCCTTCCAC AAATTGTTC GTGGATGACA CACGACCCGG GACCGAGTGC GAGACCAAT CAACCTCTCC TCCTCTCTCT ACCCATCTAA
CCTGSAATC AGTGTGAGGA GGTGACGCG CACAAGTGC CACCAACCCA CGGCTGCTCT ACCCTCTCTC CTAGCTAIGA GTTGACGCTC AGGGCCGCG
GGACCTTAAG TCACACTCCT CCCACTGCG GTGTTACCG GTGTTACCG GTCGAGGAG TGGGAGAGG GATCGATACT CAACGTCGAG TCCCSCGCG
CGATATCCTT AAGTGGCTGC AGCCAGGAAT GCTGGAAGGA TGTGTTTCA GAGGCTGCT GCGCGGCTA CTGGGGATCC CAGTGTCTT GTATGATGG
GCTATAGGAA TTCAACGAGC TCGTCTTA GCACCTTCT ACACCAAGTC TTCCGAGCA CGGGGCGGAT GACCCCTAGG GTCAGGAAAC CATACTACC
  
```

primer

guide

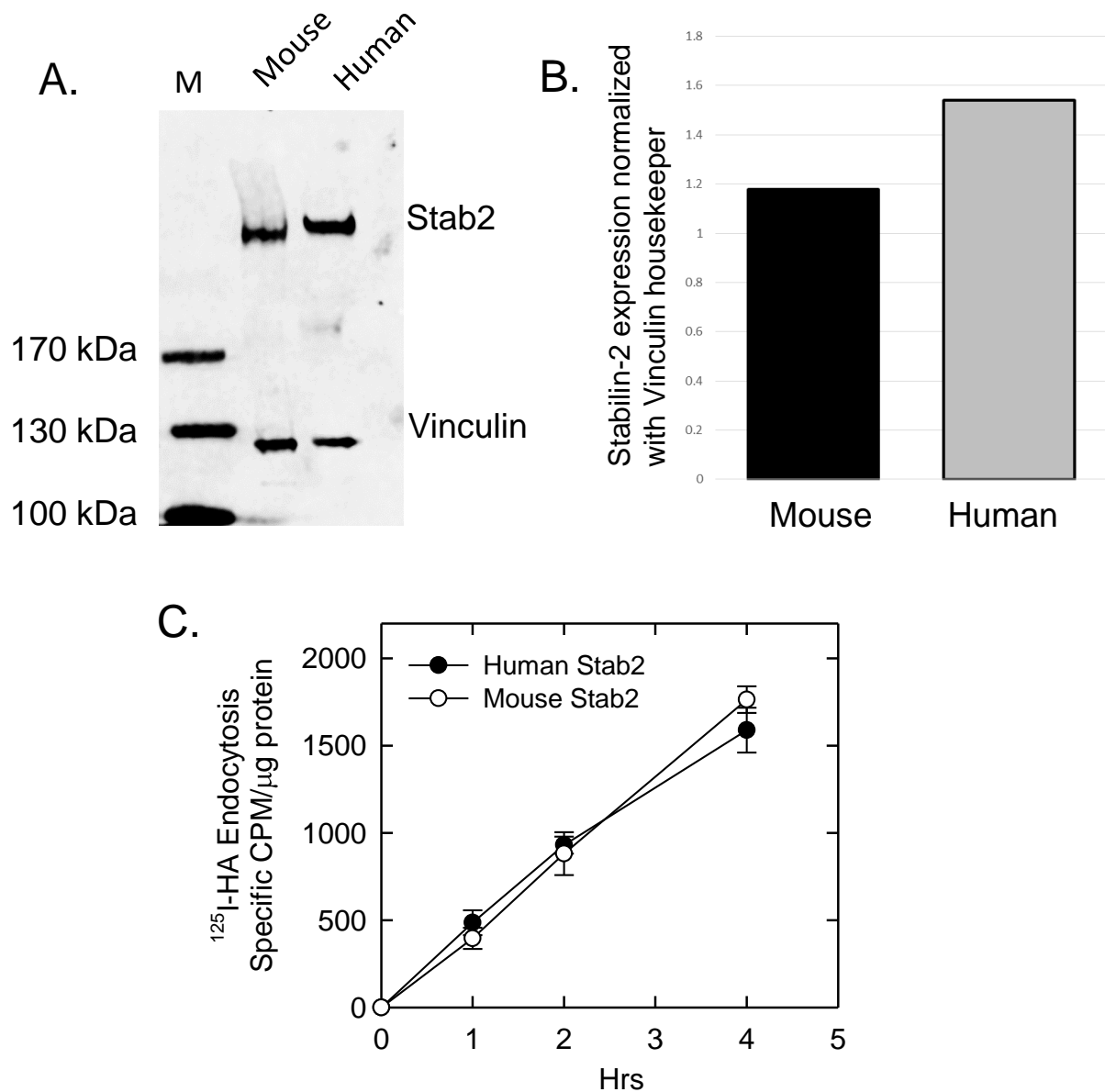
PAM

M +/+ -/- +/-



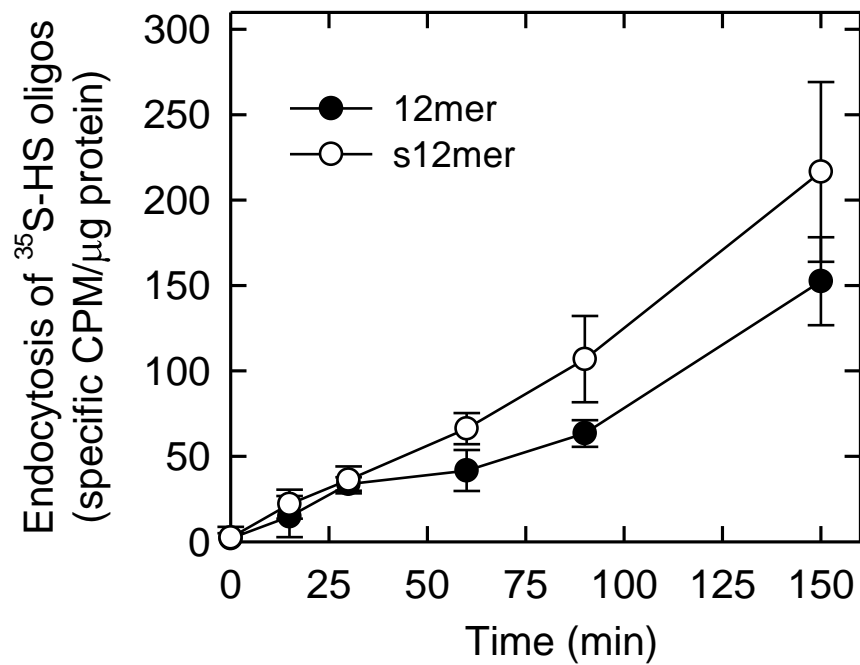
Supplemental Fig. 1: Genotype of the Stabilin-1 KO mouse line. This KO mouse line was developed by Jackson Laboratories in which the 2nd exon of the gene was deleted (dark gray region) by guide RNAs targeting flanking regions (green regions). The homozygous deletion in the mice was verified by PCR (cyan regions). The image of the gel shows WT parent (+/+), homozygous KO (-/-), and heterozygous KO (+/-) with the black arrow indicating the intact exon 2 and red arrow indicating the same amplicon with the exon 2 deletion. Only homozygous, not heterozygous Stabilin-1 KO mice were used in this study.

Supplemental Fig. 2



Supplemental Fig. 2: Characterization of the mouse Stablin-2 expressing cell line. A) 293 Flp-In cells were stably transfected with mouse Stablin-2 cDNA in pcDNA5/FRT/V5-6xHis and a clone with similar receptor expression levels to the human cell line was chosen using the criteria outline in the methods and materials section. B) Densitometry of the Stablin-2 receptor in relation to expression of the housekeeper protein, Vinculin. C) The recombinant mouse line was compared with the human line for ^{125}I -HA internalization and found to be nearly identical.

Supplemental Fig. 3



Supplemental Fig. 3: Endocytosis rates of 12mer and s12mer. Human Stabilin-2 (315-HARE) cells were plated in 24-well plates, allowed to grow up to 80% confluency over 2 days and then incubated with a single 3-*O*-sulfated or double 3-*O* sulfated dodecamer over a 2.5 hr time course. At each indicated time point, cells were washed 3x with HBSS and lysed in 0.3 mL 0.3 N NaOH. Radioactivity was evaluated with a scintillation counter and protein was measured by the Bradford assay. n=3, mean±std. deviation.